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Key Factors Determining the Behavior of Pathogens in Dry-Cured Ham after High Pressure Processing

Cristina Serra-Castelló [1], Noémie Desriac [2], Anna Jofré [1], Nicoletta Belletti [1], Louis Coroller [2] and Sara Bover-Cid [1,*]

[1] Institute of Agrifood Research and Technology (IRTA), Food Safety and Functionality Program, Finca Camps i Armet s/n, 17121 Monells, Spain

[2] Univ Brest, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE), Laboratoire Universitaire de Biodiversité et Écologie Microbienne, UMT ACTIA Alter'iX 19.03, F-29000 Quimper, France

* Correspondence: sara.bovercid@irta.cat

Abstract: High pressure processing (HPP) inactivates pathogens and increases the safety of ready-to-eat meat products. The high-pressure lethality and the behavior of the surviving cells after HPP depends on process parameters (pressure and time), microorganism and matrix characteristics. The aim of the present study was to quantify the impact of pressure level, water activity (a_w), and fat content on the behavior of *Salmonella* spp. and *Listeria monocytogenes* during refrigerated storage of dry-cured ham after high-pressure processing. *Salmonella enterica* serotype London CTC1003 and *L. monocytogenes* CTC1034 were inoculated at ca. 7 log cfu/g in dry-cured ham of different a_w (0.87–0.98), vacuum packaged, pressurized from 300 to 852 MPa for 5 min, and stored at 7 °C for up to 2 months. *Salmonella* and *L. monocytogenes* populations were monitored by plate count during the storage of the hams. The gamma concept was used to quantify the individual effects of a_w and storage temperature on the pathogen growth/no-growth behavior in pressurized dry-cured ham. The Weibull (inactivation) or Logistic (growth) primary models were fitted to the log change of pathogen levels during storage of dry-cured ham after pressurization. According to the gamma approach, the refrigeration temperature and a_w were the main factors limiting the growth of *Salmonella* and *L. monocytogenes*, respectively, in dry-cured ham. Under conditions not allowing growth, the effect of increasing pressures on the microbial inactivation depended on the a_w of dry-cured ham and the pathogen; dry-cured ham with high fat content with an $a_w \geq 0.95$ enhanced the inactivation of *Salmonella* whereas it reduced that of *L. monocytogenes*. Under conditions allowing growth of *L. monocytogenes*, the increase in a_w from 0.96 to 0.98 reduced the lag time with no apparent impact on the growth rate.

Keywords: shelf-life; food safety; *Listeria monocytogenes*; *Salmonella*; non-thermal inactivation; ready-to-eat meat products; high hydrostatic pressure

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1. Introduction

High pressure processing (HPP) is a non-thermal technology with an increasing implementation in the food industry used to enhance microbiological safety and/or render products with extended shelf-life. HPP is widely applied in meat products, representing 20–30% of overall pressurized products in the market [1]. In particular, HPP is usually used to inactivate pathogens such as *Salmonella* and *Listeria monocytogenes* in ready-to-eat (RTE) meat products once they are packed as blocks or diced or sliced convenience products. The efficacy of HPP in reducing microbial loads makes this technology particularly interesting for food-business operators to assure the accomplishment of regulations where no detection of pathogens such as *Salmonella* in RTE meat products is required [2]. Moreover, the application of HPP can be very useful to control *L. monocytogenes* in products intended to

be marketed under the umbrella of the zero-tolerance policy [3]. Within RTE meat products, dry-cured ham (DCH) is formulated with curing salts as preservatives and subsequently dried to water activity (a_w) below 0.92, which leads to DCH being considered a shelf-stable product, i.e., pathogenic microorganisms such as *Salmonella* and *L. monocytogenes* cannot grow [4–7]. However, a survey conducted on retail products of sliced and pre-packed DCH showed that 50% of the samples had an a_w above 0.92 [8]. Therefore, shelf-stability may not always be assured in terms of complying with food-safety microbiological criteria since pathogens contaminating the product may survive or even grow at a high a_w during storage. In these cases, the application of in-package lethality treatments may be needed to reduce the microbial load before storage. One of the drawbacks faced by RTE meat manufacturers when applying HPP is the enhanced pressure resistance of microorganisms in products with a low a_w, especially for products with an $a_w \leq 0.92$ such as DCH [9–11]. Therefore, the benefits in terms of pathogen-growth restriction provided by the intrinsic characteristics of DCH can turn into a limitation when applying HPP as an in-package lethality treatment aiming to eliminate pathogenic bacteria. Besides the protection of a low a_w on HPP lethality, the effects of other DCH constituents on pressure resistance and particularly the subsequent behavior of surviving cells has scarcely been evaluated. Bover-Cid et al. [9,12] studied the effect of a_w, fat, and pressure on the HPP lethality of *Salmonella* and *L. monocytogenes* in DCH. The results of these studies showed that the HPP lethality of both pathogens increased with increasing pressure and a_w of DCH. An increase in fat content did not significantly affect the lethality of *Salmonella* by HPP [12], whereas it led to a protective effect above 700 MPa for *L. monocytogenes* [9]. However, these studies did not report the influence of a_w and fat content of the DCH nor the pressure level on the subsequent behavior of *Salmonella* and *L. monocytogenes* during the storage of pressurized DCH.

Predictive microbiology, also known as quantitative microbial ecology, can be used to characterize the effect of different factors on the behavior of microorganisms in food [13]. In particular, the gamma-concept approach accounts for the individual effects of intrinsic (a_w, pH, and lactic acid) and extrinsic (storage temperature) factors and their interaction on the pathogen-growth behavior [14,15]. The aim of the present study was to continue and expand previous work on the effect of HPP on the lethality (as immediate inactivation) of *L. monocytogenes* and *Salmonella* in DCH [9,12], gaining more knowledge on the impact of the a_w and fat content of DCH and pressure level on the subsequent behavior of *Salmonella* and *L. monocytogenes* during the refrigerated storage of DCH after an HPP treatment. To do so, a quantitative study based on the determination of kinetic parameters was used to characterize the behavior of *Salmonella* and *L. monocytogenes* in DCH after HPP.

2. Material and Methods

2.1. Bacterial Strains

The bacterial strains used in the present study were *Salmonella* serovar London CTC1003 and *L. monocytogenes* strain CTC1034. Both strains were originally isolated from dry-cured meat products and have been used in previous studies dealing with the application of HPP in meat products [9,12]. The inoculums were prepared as in Bover-Cid et al. [9]. Briefly, stock cultures (stored in 20% glycerol at −80 °C) were transferred to 10 mL Brain Heart Infusion (BHI, from DB, NJ, USA) broth and incubated at 37 °C for 7 h. A second subculture was performed by transferring the first culture into a second tube of BHI and incubated at 37 °C for 18 h. An appropriate volume of this overnight culture was properly diluted to finally obtain a high inoculum level of ca. 10^7 cfu/g in DCH to ensure quantifiable levels were obtained after HPP.

2.2. Experimental Designs and Preparation of the Samples

The experimental layout of the experimental designs is shown in Figure 1. Firstly, a central composite design (CCD) with three variables (a_w, fat content, and pressure) and five levels, with an a_w ranging from 0.86–0.94, fat from 10–50%, and pressure level from 347–852 MPa, as described in Bover-Cid et al. [9,12], was followed. Secondly, to characterize

subsequent behavior of the pathogens under DCH a_w values around the growth/no-growth interface, a full factorial design (FFD) with 2 variables (a_w and pressure) was conducted in DCH with an a_w of 0.94–0.98 and with a fixed fat content of 30%. The factors and levels of the design were selected to cover a wide range of physicochemical characteristics of DCH [16].

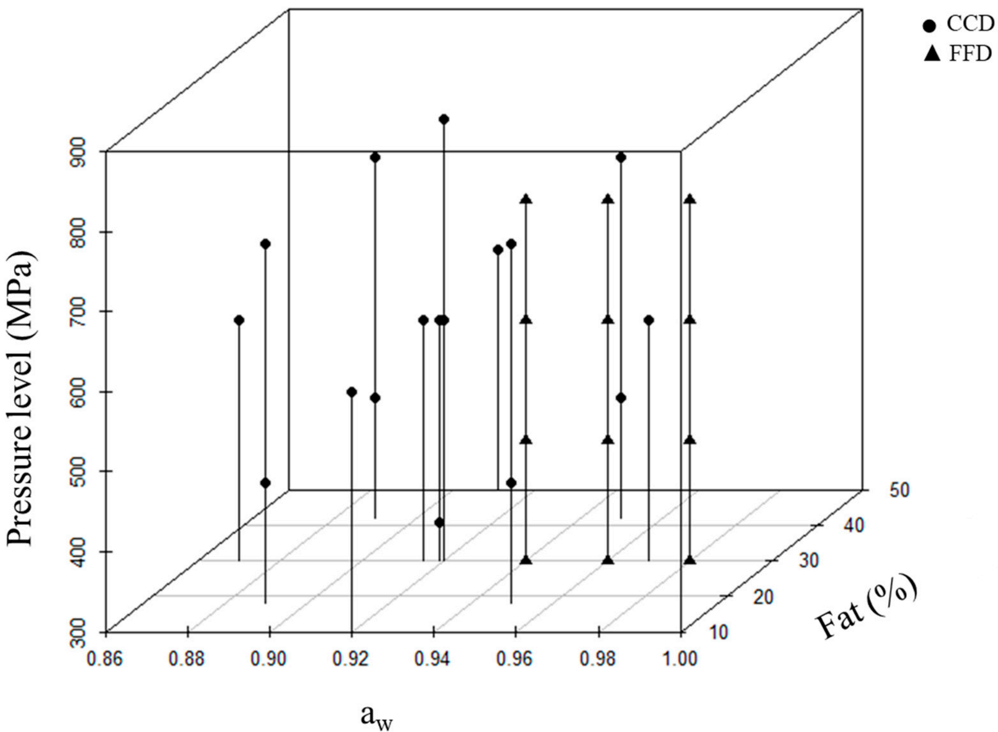

Figure 1. Scatterplot showing the a_w and fat content of the dry-cured ham (DCH) and pressure levels included in the experimental conditions of the central composite design (CCD) and the full factorial design (FFD).

DCHs were aseptically deboned in the laboratory. The lean part (with an a_w of 0.85 and 6.7% fat) was aseptically separated from the fat part, and each part was separately minced under aseptic conditions (minced lean showing pH 5.7 and 5000 ppm of water-phase lactic acid from endogenous origin). DCH matrices with the a_w and fat content adjusted in accordance with the target values of the experimental-design trials were prepared as described in Bover-Cid et al. [9,12]. Briefly, to adjust the a_w of the product, the appropriate volume of distilled water was added to the minced lean part, mixed, and equalized until homogenization to reach the target a_w. The inoculum was added to the distilled water immediately before mixing with the minced lean part. Afterwards, the proper quantity of minced fat corresponding to each trial was added to the inoculated lean samples. The actual a_w of the samples was verified with Aqualab™ equipment (Series 3, Decagon Devices Inc., Pullman, WA, USA). The DCH was distributed in 15 g-samples and vacuum-packaged in PET/PE plastic bags (with oxygen permeability < 50 $cm^3/m^2/24$ h and water vapor permeability < 15 $mg/m^2/24$ h; Sacoliva S.L., Barcelona, Spain). For each trial, two DCH sample replicates were prepared.

2.3. HPP and Subsequent Storage of DCH

Samples were pressurized at the target pressure according to the corresponding trial of the experimental designs, which were in the range of 347 to 852 MPa. All treatments were applied for 5 min and with an initial fluid temperature of 15 °C. For pressures up to 600 MPa, Wave 6000 Hiperbaric (Burgos, Spain) equipment was used. For pressures above 600 MPa, Thiot ingeniere—Hiperbaric (Bretenoux, France—Burgos, Spain) equipment was

used. The pressure come-up rate was on average 220 MPa/min and pressure release was almost immediate (<2 s). After HPP, samples were stored at 7 °C for up to 60 days and were periodically taken for bacterial enumeration. Samples were stored at 7 °C as recommended and at foreseeable storage conditions for ready-to-eat meat products [17–19].

2.4. Microbiological Analysis

Samples were homogenized (1/10 dilution) with tryptic soy broth with 0.6% yeast extract (TSBYE; DB, NJ, USA) in a Masticator Classic (IUL S.A., Barcelona, Spain) for 1 min and subsequently 10-fold diluted in 0.1% Bacto Peptone (Difco Laboratories, Detroit, MI, USA) with 0.85% NaCl (Merck, Darmstadt, Germany). For the periodical enumeration of *Salmonella*, homogenates were plated on Brilliant Green Agar (BGA; Difco Laboratories, Detroit, MI, USA) and incubated at 37 °C for 24–48 h. For *L. monocytogenes*, homogenates were plated on the selective and differential medium Chromogenic Listeria Agar (CLA; Oxoid Basingstoke, UK) and incubated at 37 °C for 48 h. For expected counts below the limit of quantification, i.e., 4 cfu/g (resulting from platting 4 mL of homogenate in a 14 cm diameter plate), the presence of both pathogens in 15 g test samples was determined by enrichment of the homogenates at 37 °C for 48 h. The enriched homogenates were streaked on selective media (BGA for *Salmonella* and CLA for *L. monocytogenes*) and incubated at 37 °C for 24–48 h. Presumptive colonies were confirmed by PCR [20].

2.5. Statistical Analysis and Mathematical Modeling of the Pathogen Behavior during the Storage of the DCH after the HPP

The statistical analysis and mathematical modeling were conducted on data transformed into log change (log N/N_0), i.e., the decimal logarithm of the pathogen concentration at each sampling point minus the concentration of the pathogen at the beginning of the storage (immediately after the HPP). Values of the log change > 0.5 log units were considered growth behavior, log changes <−0.5 log were considered inactivation behavior, and log changes between −0.5 and 0.5 were considered not microbiologically relevant changes [18] and are termed "survival" in this article.

Principal component analysis (PCA) was conducted to provide a general overview of the dynamics along the storage of the pathogen in terms of changes in the concentration (log change) after HPP for the different combination of conditions. To perform the PCA, the *estim_ncpPCA* and *imputePCA* functions from the *missMDA* package of R software [21] were used to deal with the missing values, i.e., when the pathogen was not detected. The PCA with the confidence ellipses around the categoric variables (a_w, fat, and pressure level) at a confidence level of 0.95 was obtained with the *PCA* and *plotellipses* functions from the *FactoMinerR* package of R software [21]. Statistical differences in microbial log change (log N/N_0) along the storage time between trials were assessed through an ANOVA test followed by a Tukey's honestly significant difference test. For this, the *aov* function from the *stats* package and the *TukeyHSD* function from the *agricolae* package of the R software [21] were used.

2.5.1. Estimation of Growth/No-Growth Behavior

To assess whether the experimental environmental conditions (i.e., intrinsic parameters of DCH and storage temperature) of each trial would support the growth of *L. monocytogenes* and *Salmonella* during the storage of the DCH after HPP, the gamma-concept approach was applied [14,15,22]. The overall effect of the combination of the most relevant environmental factors influencing the growth (i.e., intrinsic: a_w, pH, lactic-acid concentration; and extrinsic: storage temperature) was estimated by calculating the overall gamma product (Γ. Equation (1)), including the interaction factor.

$$\Gamma = \prod_{i=1}^{k} \gamma_X(X_i) \cdot \xi \tag{1}$$

where (X_i) is defined by the physico-chemical properties of the ham during the storage (e.g., pH, a_w, lactic acid, and temperature). The individual effect of each environmental factor (X) on the pathogen growth is described by the individual gamma factor γ_X, whereas ξ is the interaction between factors. The γ_X and ξ values can vary from 0 to 1, with 0 indicating that growth is depleted by the environmental factor at a level of X_i or the interaction ξ and 1 indicating that the growth potential is optimal for this particular environmental factor [15]. The detailed procedure and cardinal values used for the calculation of γ_X and ξ values are described in Figure S1 and Table S1.

The growth behavior was defined based on the overall product of gamma factors with their interaction, being considered no growth when the output of Equation (1) was zero ($\Gamma = 0$) and growth when the model output was higher than zero ($\Gamma > 0$) [15]. For no-growth conditions ($\Gamma = 0$) the inactivation behavior was further explored according to Section 2.5.2, whereas when growth conditions were observed the growth kinetic parameters were characterized according to Section 2.5.3.

2.5.2. Non-Thermal Inactivation Kinetic Parameters during the Storage of DCH after HPP

The Weibull model (Equation (2)) was used to estimate the kinetic parameters describing the non-thermal inactivation of the pathogens during the refrigerated storage of DCH after HPP.

$$log\left(\frac{N}{N_0}\right) = -\left(\frac{t}{\delta}\right)^p \tag{2}$$

where N is the number of cells at time t and N_0 is the number of cells at the beginning of the storage time; $log\ (N/N_0)$ is the inactivation in log reduction (log units) at a given time (t) of the storage, being equal to 0 at storage time 0; δ is the time (days) necessary to obtain the first log reduction; and p is the shape parameter. Model fitting was carried out using the *nls2* package of the R software [21].

2.5.3. Estimation of Growth Kinetic Parameters during the Storage of DCH after HPP

The primary Logistic growth model with delay (Equation (3), [23]) was used to estimate the growth kinetic parameters of the pathogens during the refrigerated storage of DCH after HPP. The model was fitted to the log change data (i.e., log N_t/N_0) using the *nls2* package of the R software [21].

$$\begin{aligned} &\text{For } t < \lambda,\ \log\left(\frac{N_t}{N_0}\right) = 0 \\ &\text{For } t \geq \lambda,\ \log\left(\frac{N_t}{N_0}\right) = \log\left(\frac{MGP}{1+\left(\frac{MGP}{N_0}-1\right)\cdot(\exp(-\mu_{max}\cdot(t-\lambda)))}\right) \end{aligned} \tag{3}$$

where N_0 is the concentration of the pathogen (cfu/g) at time zero, N_t is the concentration of the pathogen (cfu/g) at a given time (t), MGP is the maximum growth potential (maximum bacterial increase in log units), λ is the lag time (days); μ_{max} is the maximum specific growth rate (h^{-1}), and t is the storage time (days).

3. Results and Discussion

3.1. *Salmonella* Behavior in DCH during Storage after HPP

The results of the challenge test showed that *Salmonella* was not able to grow in any of the conditions assessed (Figure 2). The results agreed with those obtained through the application of the gamma concept, accounting for the individual effects of intrinsic (a_w, pH, and lactic acid) and extrinsic (storage temperature) factors and their interaction on *Salmonella* behavior, where no growth ($\Gamma = 0$) was predicted to occur in DCH with a $a_w < 0.98$ (Table 1). Growth of *Salmonella* was predicted at $a_w = 0.98$ but the Γ value was very close to zero ($\Gamma = 0.0002$; trials 29–32; Table 1). The main contributing growth-inhibition factor was the storage temperature ($\gamma_T = 0.005$, Table S2) since the storage temperature applied (7 °C) was close to the minimum growth temperature of *Salmonella* (Table S1). Results of challenge test showed that under the non-growing conditions, the viability of *Salmonella*

was compromised in all the trials, resulting in a progressive log reduction (inactivation) of the pathogen load along the refrigerated storage (Figure 2, Table 1). The results of the PCA analysis showed that 99.73% of the variability could be represented in a two-dimensional space (Figure 3). The same length of the arrows represented in the correlation circle shows that all the sampling times contributed highly to a similar extent to explaining the variability in the *Salmonella* log change data during storage (Figure 3a). In dimension 1, the horizontal axis explained 92.58% of the variability of the *Salmonella* log change data and all the arrows pointed in the same direction, indicating a high correlation among sampling times. The confidence ellipses grouped trials with pressure < 600 MPa on the right and trials with pressure > 600 MPa on the left of the PCA graph (Figure 3d), suggesting an important impact of pressure level on *Salmonella* inactivation along the storage of DCH, which could be related to the high lethality of > 600 MPa treatments.

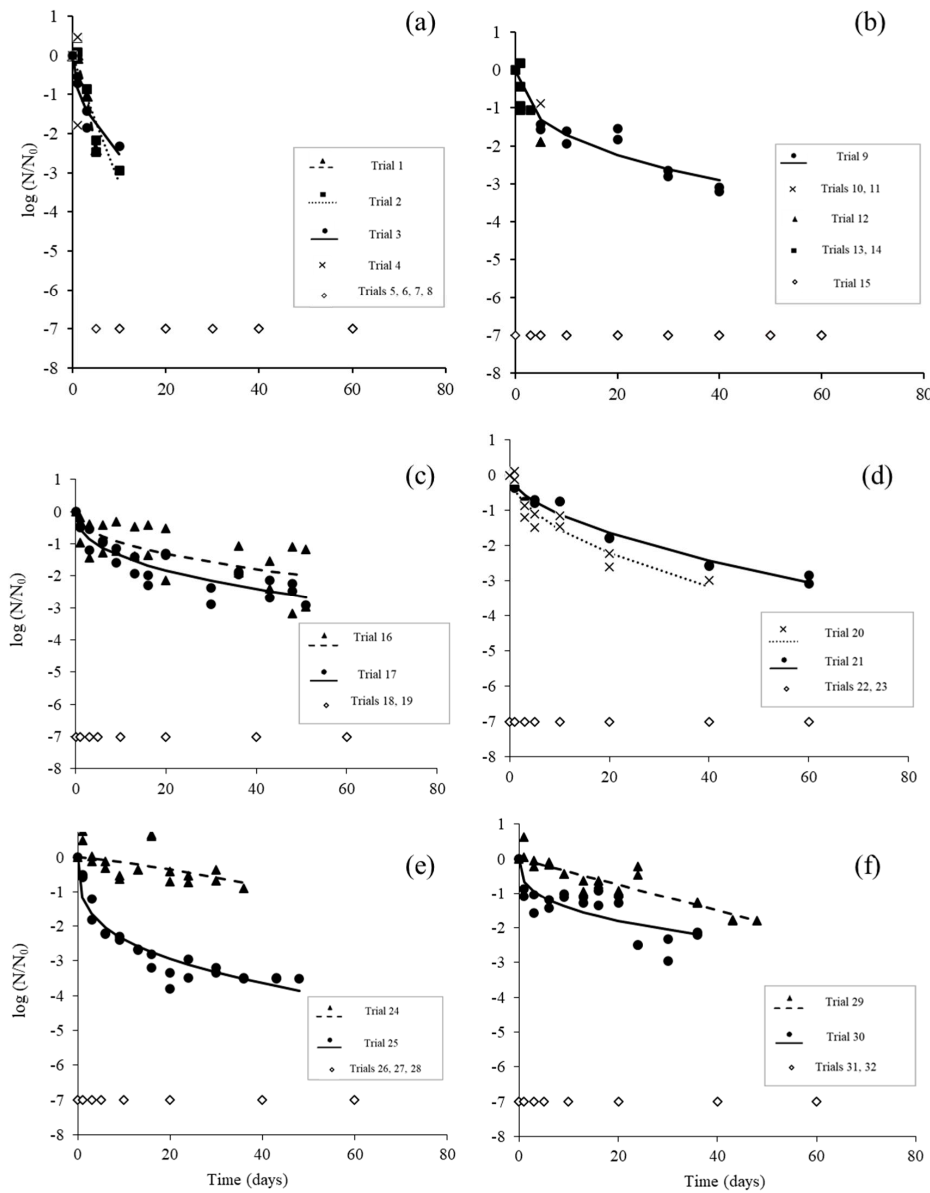

Figure 2. *Salmonella* survival and inactivation during storage at 7 °C of pressurized dry-cured ham (DCH) with an a_w of (**a**) 0.870–0.915, (**b**) 0.919–0.920, (**c**) 0.940, (**d**) 0.950, (**e**) 0.960, and (**f**) 0.980. Symbols correspond to log change values and lines to the fit of the Weibull model (Equation (2)) to the data. Empty symbols correspond to replicates where the pathogen was not detected.

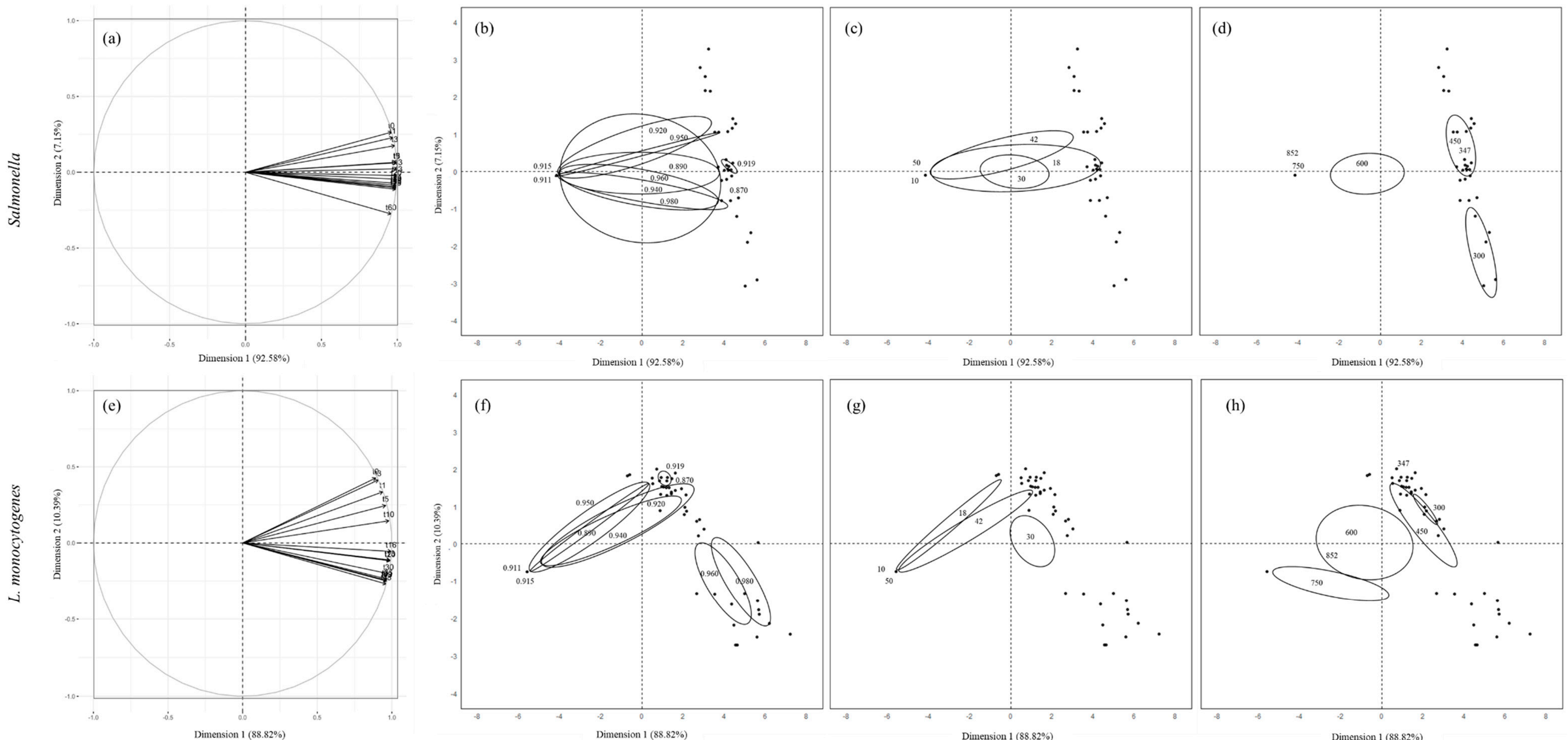

Figure 3. Results of the principal component analysis (PCA) performed on the log change values of *Salmonella* (**a**–**d**) and *L. monocytogenes* (**e**–**h**) observed during the storage of pressurized dry-cured ham (DCH) at 7 °C. (**a**,**e**) show the correlation circles with the relationship between all the sampling times (days) on the log change data of *Salmonella* and *L. monocytogenes*. In (**b**–**d**,**f**–**h**), the points represent the scores for the first two dimensions of PCA corresponding to each replicate of the 32 trials performed and the ellipses correspond to confidence ellipses at a confidence level of 0.95 around the categoric variables a_w (**b**,**f**), % of fat content (**c**,**g**), and pressure level in MPa (**d**,**h**).

Table 1. *Salmonella* concentration immediately after high pressure processing (HPP) in the different trials and estimated kinetic-parameter values resulting from fitting the primary inactivation model to the *Salmonella* inactivation counts observed during the storage of pressurized dry-cured ham (DCH) at 7 °C.

Trial	DCH Characteristics		HPP (MPa)	Concentration after HPP (log cfu/g) [a]	Observed Behavior during Storage [b]	Predicted G/NG (Γ) [d]	Inactivation Kinetic Parameters [e]		Goodness of Fit [f]		
	a_w	Fat (%)					δ (Days)	p	n	par	RMSE
1	0.870	30	600	3.71 ± 0.04	I	0 (NG)	3.03 ± 0.10	1.80 ± 0.13	7	2	0.091
2	0.890	18	450	3.55 ± 0.17	I	0 (NG)	2.90 ± 0.76	0.95 ± 0.25	9	2	0.538
3	0.890	42	450	2.92 ± 0.11	I	0 (NG)	1.48 ± 0.35	0.49 ± 0.08	7	2	0.215
4	0.890	18	750	2.69 ± 0.79	I [c]	0 (NG)	-	-	-	-	-
5	0.890	42	750	0.90 ± 0.43	I [c]	0 (NG)	-	-	-	-	-
6	0.911	50	600	1.66 ± 0.26	I [c]	0 (NG)	-	-	-	-	-
7	0.915	30	600	1.39 ± 0.55	I [c]	0 (NG)	-	-	-	-	-
8	0.915	30	600	1.80 ± 0.14	I [c]	0 (NG)	-	-	-	-	-
9	0.919	30	347	5.76 ± 0.17	I	0 (NG)	2.43 ± 1.07	0.38 ± 0.06	16	2	0.365
10	0.919	30	600	2.78 ± 0.07	I [c]	0 (NG)	-	-	-	-	-
11	0.919	30	600	3.03 ± 0.20	I [c]	0 (NG)	-	-	-	-	-
12	0.920	10	600	2.57 ± 0.22	I [c]	0 (NG)	-	-	-	-	-
13	0.920	30	600	2.72 ± 0.22	I [c]	0 (NG)	-	-	-	-	-
14	0.920	30	600	2.38 ± 0.66	I [c]	0 (NG)	-	-	-	-	-
15	0.920	30	852	<DL	K	0 (NG)	-	-	-	-	-
16	0.940	30	300	7.10 ± 0.63	I	0 (NG)	10.76 ± 4.31	0.44 ± 0.14	24	2	0.643
17	0.940	30	450	4.74 ± 0.03	I	0 (NG)	4.56 ± 1.23	0.41 ± 0.06	26	2	0.356
18	0.940	30	600	<DL	K	0 (NG)	-	-	-	-	-
19	0.940	30	750	<DL	K	0 (NG)	-	-	-	-	-
20	0.950	18	450	4.64 ± 0.03	I	0 (NG)	8.18 ± 0.96	0.56 ± 0.04	16	2	0.186
21	0.950	42	450	3.70 ± 0.15	I	0 (NG)	4.42 ± 0.98	0.52 ± 0.07	13	2	0.316
22	0.950	18	750	<DL	K	0 (NG)	-	-	-	-	-
23	0.950	42	750	<DL	K	0 (NG)	-	-	-	-	-
24	0.960	30	300	6.48 ± 0.05	I	0 (NG)	46.06 ± 5.97	1.24 ± 0.43	25	2	0.409
25	0.960	30	450	4.40 ± 0.01	I	0 (NG)	0.59 ± 0.23	0.31 ± 0.03	26	2	0.336
26	0.960	30	600	<DL	K	0 (NG)	-	-	-	-	-
27	0.960	30	600	<DL	K	0 (NG)	-	-	-	-	-
28	0.960	30	750	<DL	K	0 (NG)	-	-	-	-	-
29	0.980	30	300	6.30 ± 0.01	I	2.16×10^{-4} (G)	26.75 ± 1.98	1.00 ± 0.15	24	2	0.267
30	0.980	30	450	3.63 ± 0.09	I	2.16×10^{-4} (G)	3.48 ± 1.66	0.33 ± 0.09	22	2	0.457
31	0.980	30	600	<DL	K	2.16×10^{-4} (G)	-	-	-	-	-
32	0.980	30	750	<DL	K	2.16×10^{-4} (G)	-	-	-	-	-

[a] Mean ± standard deviation of *Salmonella* concentration immediately after HPP. Conditions where the pathogen concentration after HPP was below the detection limit are indicated with <DL. [b] I: inactivation behavior (observed log change <-0.5 log units); K: the application of HPP resulted in the total inactivation of the pathogen in the sample unit and/or the pathogen was not able to recover viability during storage. [c] Few quantification points before *Salmonella* was inactivated to concentrations below the detection limit or no detection of the pathogen during storage. Kinetic parameters could not be estimated. [d] Growth/no-growth (G/NG) boundary as predicted by the gamma concept (Γ). For each DCH with a different a_w value, Γ was calculated considering a storage temperature of 7 °C, pH of DCH of 5.7, and lactic-acid content of DCH of 5000 ppm in the water phase. [e] The Weibull model was fitted to log change data (Equation (2)) to estimate the inactivation kinetic parameters. δ: time (days) for the first log reduction during storage; p: shape of the inactivation curve. The estimate ± standard error is provided. [f] n: number of data points ($\log N/N_0$) included for fitting; par: parameters estimated in the model; RMSE: root mean square error.

Specifically, the pressurization of DCH at $\geq$ 600 MPa resulted in a lethality of *Salmonella* to levels below the detection limit in DCH with an a_w > 0.92 from immediately after HPP to the end of the storage (trials 18–19, 22–23, 26–28, 31–32; Table 1). In DCH with an $a_w \leq 0.92$ (trials 4–8, 10–14; Table 1), due to the protective effect of a low a_w in front of HPP or piezo-protection [12], *Salmonella* could be enumerated. However, after HPP the remaining levels were low and decreased below the detection limit just after 1 day of storage, thus not allowing for the estimation of inactivation kinetic parameters (trials 4–8, 10–14; Table 1). In this framework, Stollewerk et al. [24] observed that the levels of *Salmonella* after the application of HPP at 600 MPa for 5 min in smoked DCH (pH of 5.87 and a_w of 0.93) were low and progressively decreased below the detection limit after the storage of DCH at 4 °C for 38 days and afterwards at 8 °C for 18 days. The longer survival of *Salmonella* in DCH observed by Stollewerk et al. [24] compared to the results obtained in the present study could be partially attributed to the lower storage temperature. In this respect, Serra-Castelló et al. [6] quantified that the storage of DCH at 4 °C favors the survival of *Salmonella* in non-pressurized DCH compared to the storage of the product at 7 °C. On the other hand, the application of pressure levels < 600 MPa led to *Salmonella* concentrations above the detection limit, allowing inactivation kinetics to be characterized along the storage of the DCH with different intrinsic characteristics (a_w and fat) (trials 1–3, 9, 16–17, 20–21, 24–25, 29–30; Table 1; Figure 2).

The increase in the pressure level applied from 300 to 450 MPa resulted in enhanced *Salmonella* inactivation (shorter δ parameter) during the refrigerated storage of DCH (trials 16–17, 24–25 and 29–30; Table 1; Figure 2), though its impact on the *Salmonella* inactivation kinetics curve was only statistically significant (p-value < 0.05) in DCH with an $a_w \geq 0.96$ (trials 24–25, 29–30; Figure 2). A higher content of fat (42%) in DCH seemed to enhance the inactivation of the pathogen during the refrigerated storage after HPP in DCH with an a_w of 0.89 and especially with an a_w of 0.95 (trials 2–3, 20–21; Table 1), though it was not statistically significant.

3.2. L. monocytogenes Behavior in DCH during Storage after HPP: Growth/No Growth

The results of challenge test showed that *L. monocytogenes* was able to grow in DCH with an a_w of 0.96 regardless of the HPP level applied, whereas growth was not observed in DCH with a lower a_w (Figure 4). Contrary to *Salmonella*, the output of the gamma approach showed that temperature was not the main limiting factor for the pathogen's growth (γ_T = 0.055, Table S2), as *L. monocytogenes* is a psychrotrophic microorganism able to grow at temperatures slightly below 0 °C [25]. The quantification of the individual effects of the intrinsic (a_w, pH, and lactic acid concentration) and extrinsic (storage temperature) factors and their interactions on the *L. monocytogenes* behavior through the gamma concept showed that, within the experimental domain of the present study, the growth/no-growth boundary predictions for this pathogen depended on the a_w, with the DCH with an a_w value of 0.95 being the predicted boundary value for *L. monocytogenes* growth (Table 2). A value of 0.92 is considered the minimum growth limit for *L. monocytogenes* when no other stressing factors are present [25]. However, when a low a_w is combined with other factors such as low temperature, its bacteriostatic effect is enhanced and the minimum growth limit decreases. In this respect, the microbiological criteria for foodstuffs [2] established that foods with an a_w < 0.92 are automatically considered unable to support the growth of the pathogen. According to this regulation, other categories of products can also belong to this category, subject to scientific justification. The growth/no-growth model predicted growth in DCH with an a_w = 0.95 with a very small Γ value ($\Gamma = 3.14 \times 10^{-3}$) and no growth of *L. monocytogenes* was observed when it was pressurized at 450 or 750 MPa (Trials 52–55, Table 2), indicating that the growth/no-growth boundary of the pathogen in pressurized DCH was also limited by other factors not considered in the calculation of the Γ factor, which could also include the potential injury caused by HPP on *L. monocytogenes* cells. Therefore, the present study provides scientific evidence to justify that the pressurized

DCH with an a_w > 0.92, up 0.95, can belong to the category not supporting the growth of *L. monocytogenes*.

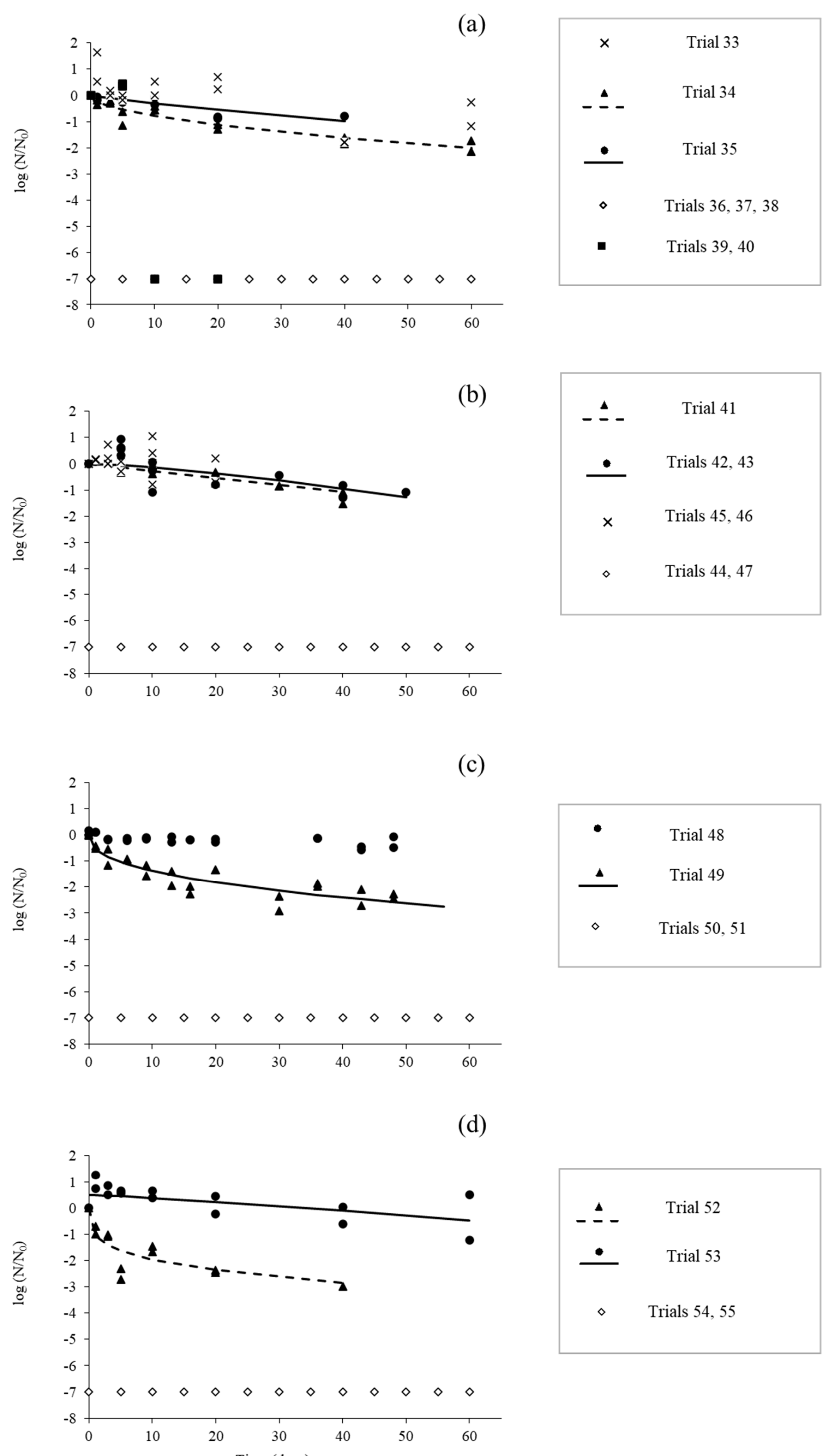

Figure 4. *L. monocytogenes* survival and inactivation during the storage at 7 °C of pressurized dry-cured ham (DCH) with an a_w (**a**) 0.870–0.915, (**b**) 0.919–0.920, (**c**) 0.940, and (**d**) 0.950. Symbols correspond to observed counts and lines to the fit of the Weibull model (Equation (2)) to data. Empty symbols correspond to replicates where the pathogen was not detected.

Table 2. *L. monocytogenes* concentration immediately after high pressure processing (HPP) in the different trials and estimated parameter values resulting from fitting the primary models to the *L. monocytogenes* inactivation/growth counts observed during the storage of pressurized dry-cured ham (DCH) at 7 °C.

Trial	DCH Characteristics		HPP (MPa)	Concentration after HPP (log cfu/g) [a]	Observed Behavior during Storage [b]	Predicted G/NG (Γ) [d]	Inactivation Parameters [e]		Growth Parameters [f]			Goodness of Fit [f,g]		
	a_w	Fat (%)					δ (Days)	*p*	λ (d)	μ_{max} (h^{-1})	*MGP* (log)	n	par	RMSE
33	0.870	30	600	1.78 ± 0.17	S	0 (NG)	-	-	-	-	-	-	-	-
34	0.890	18	450	4.34 ± 0.05	I	0 (NG)	15.74 ± 2.51	0.52 ± 0.08	-	-	-	15	2	0.251
35	0.890	42	450	4.69 ± 0.06	I	0 (NG)	40.28 ± 3.55	0.85 ± 0.15	-	-	-	15	2	0.191
36	0.890	18	750	<DL	K	0 (NG)	-	-	-	-	-	-	-	-
37	0.890	42	750	<DL	K	0 (NG)	-	-	-	-	-	-	-	-
38	0.911	50	600	<DL	K	0 (NG)	-	-	-	-	-	-	-	-
39	0.915	30	600	1.57 ± 0.38	I [c]	0 (NG)	-	-	-	-	-	-	-	-
40	0.915	30	600	1.81 ± 0.05	I [c]	0 (NG)			-	-	-			
41	0.919	30	347	5.83 ± 0.26	I	0 (NG)	37.57 ± 2.92	0.96 ± 0.19	-	-	-	14	2	0.227
42	0.919	30	600	1.96 ± 0.17	I	0 (NG)	41.84 ± 7.79	1.35 ± 0.80 [ns]	-	-	-	15	2	0.501
43	0.919	30	600	2.22 ± 0.12	I	0 (NG)			-	-	-			
44	0.920	10	600	<DL	K	0 (NG)	-	-	-	-	-	-	-	-
45	0.920	30	600	1.68 ± 0.17	I [c]	0 (NG)	-	-	-	-	-	-	-	-
46	0.920	30	600	1.15 ± 0.21	I [c]	0 (NG)			-	-	-			
47	0.920	30	852	<DL	K	0 (NG)	-	-	-	-	-	-	-	-
48	0.940	30	300	6.81 ± 0.12	S	0 (NG)	-	-	-	-	-	-	-	-
49	0.940	30	450	5.80 ± 0.02	I	0 (NG)	4.86 ± 1.17	0.40 ± 0.05	-	-	-	24	2	0.348
50	0.940	30	600	<DL	K	0 (NG)	-	-	-	-	-	-	-	-
51	0.940	30	750	<DL	K	0 (NG)	-	-	-	-	-	-	-	-
52	0.950	18	450	5.61 ± 0.07	I	3.14×10^{-3} (G)	0.81 ± 0.53	0.27 ± 0.05	-	-	-	15	2	0.432
53	0.950	42	450	2.15 ± 0.21	I	3.14×10^{-3} (G)	61.44 ± 28.77	1.18 ± 1.38 [ns]	-	-	-	15	2	0.792
54	0.950	18	750	<DL	K	3.14×10^{-3} (G)	-	-	-	-	-	-	-	-
55	0.950	42	750	<DL	K	3.14×10^{-3} (G)	-	-	-	-	-	-	-	-
56	0.960	30	300	7.02 ± 0.05	G	1.34×10^{-2} (G)	-	-	5.76 ± 1.55	0.011 ± 0.003	1.34 ± 0.08	26	3	0.142
57	0.960	30	450	2.10 ± 1.70	G	1.34×10^{-2} (G)	-	-	6.00 ± 2.97	0.038 ± 0.013	4.80 ± 0.40	24	3	1.644
58	0.960	30	600	<DL	G	1.34×10^{-2} (G)	-	-	NA	0.022 ± 0.003	7.05 ± 0.31	28	2	0.866
59	0.960	30	600	<DL	G	1.34×10^{-2} (G)								
60	0.960	30	750	<DL	G	1.34×10^{-2} (G)	-	-	NA	$0.088 \pm 7.541 \times 10^{3}$ [ns]	6.20 ± 0.71	12	2	1.560
61	0.980	30	300	6.81 ± 0.10	G	2.78×10^{-2} (G)	-	-	NA	0.021 ± 0.003	1.36 ± 0.04	22	2	0.163

Table 2. *Cont.*

Trial	DCH Characteristics		HPP (MPa)	Concentration after HPP (log cfu/g) [a]	Observed Behavior during Storage [b]	Predicted G/NG (Γ) [d]	Inactivation Parameters [e]		Growth Parameters [f]			Goodness of Fit [f,g]		
	a_w	Fat (%)					δ (Days)	p	λ (d)	μ_{max} (h^{-1})	*MGP* (log)	n	par	RMSE
62	0.980	30	450	0.98 ± 0.11	G	2.78×10^{-2} (G)	-	-	NA	0.033 ± 0.003	6.89 ± 0.19	22	2	0.526
63	0.980	30	600	<DL	G	2.78×10^{-2} (G)	-	-	NA	0.021 ± 0.002	6.95 ± 0.34	17	2	1.327
64	0.980	30	750	<DL	G	2.78×10^{-2} (G)	-	-	NA	0.039 ± 0.014	6.96 ± 0.74	13	2	0.744

[ns] Parameter estimates not statistically significant. [a] Mean ± standard deviation of *L. monocytogenes* concentration immediately after HPP. Conditions where the pathogen concentration after HPP was below the detection limit are indicated with <DL. [b] S: *L. monocytogenes* survived without growth or inactivation (observed log change between −0.5 and 0.5 log units); I: inactivation (observed log change < −0.5 log units); K: the application of HPP resulted in the total inactivation of the pathogen in the sample unit and/or the pathogen was not able to recover viability during the storage; G: growth (observed log change > 0.5 log units). [c] Few quantification points before *L. monocytogenes* inactivated to concentration below the detection limit or no detection of the pathogen during storage. Kinetic parameters could not be estimated. [d] Growth/no-growth (G/NG) boundary as predicted by the gamma concept (Γ). For each DCH with different a_w value, Γ was calculated considering a storage temperature of 7 °C, pH of DCH of 5.7, and lactic-acid content of DCH of 5000 ppm in the water phase. [e] For conditions not supporting growth that caused a loss of *L. monocytogenes* viability, i.e., inactivation, the Weibull model was fitted to log change data (Equation (2)) to estimate the inactivation kinetic parameters. δ: time (days) for the first log reduction during storage; p: shape of the inactivation curve. The estimate ± standard error is provided. [f] For conditions supporting growth, the logistic growth model without and with delay (Equation (3)) was fitted to log change data to estimate the growth kinetic parameters. λ is the lag time (days), μ_{max} is the maximum specific log increase rate (h^{-1}); MGP is the maximum growth potential (maximum bacterial increase in log units). The estimate ± standard error is provided. [g] n: number of log change data points (log N/N_0) included for fitting; par: parameters estimated in the model; RMSE: root mean square error.

The results of the PCA showed that 99.21% of the variance in the log change data of *L. monocytogenes* could be represented in a two-dimensional space (Figure 3). The same length of the arrows represented shows that all the sampling times highly contributed to some extent to explaining the variability in the *L. monocytogenes* log change data during storage (Figure 3e). In dimension 1, all the arrows pointed to the right, indicating that the sampling times were correlated. The confidence ellipses grouped trials with pressure < 600 MPa on the right and trials with pressure > 600 MPa on the left of the PCA graph (Figure 3h), suggesting an important impact of pressure level on *L. monocytogenes* inactivation along the storage of DCH. Moreover, confidence ellipses also grouped trials with an $a_w \geq 0.96$ and trials with an $a_w < 0.96$ (Figure 3f), which was correlated with the observed growth of the pathogen (log change > 0) in DCH with an $a_w \geq 0.96$ and no growth (log change ≤ 0) with an $a_w < 0.96$ (Table 2).

3.2.1. *L. monocytogenes* No-Growth Conditions: Survival and Inactivation during Storage

No growth of *L. monocytogenes* was predicted to occur ($\Gamma = 0$) in products with an $a_w < 0.95$ (Table 2), which agreed with the observed results, where survival (no microbiologically relevant change, trials 33 and 48) or inactivation of *L. monocytogenes* was observed during the storage of DCH (trials 34–47 and 49–51; Table 2; Figure 4).

Trials involving HPP at 750–852 MPa had a strong lethal effect (*L. monocytogenes* was not detected), not allowing subsequent monitoring (trials 36–38, 44–47, 50–51, 54–55; Table 2; Figure 4). For pressure levels < 600 MPa, the concentration of *L. monocytogenes* after the HPP and its subsequent behavior was affected by the pressure level applied as well as the DCH's characteristics (a_w value and fat content). In DCH with an a_w of 0.87 pressurized at 600 MPa (Trial 33; Table 2; Figure 4a) the levels of *L. monocytogenes* after HPP were variable and close to the quantification level, with no detection of the pathogen in 12.5% of the samples along the storage. Overall, no relevant change (survival without inactivation) can be associated with these trial conditions. The survival of *L. monocytogenes* could be the consequence of the piezo-protection effect exerted by low a_w on the lethality of the pathogen by HPP. Indeed, this would result in fewer injured *L. monocytogenes* cells due to its role in protein stabilization, which prevents protein denaturation and cell death during HPP [26], and which in turn could contribute to keeping the pathogen viability after HPP.

L. monocytogenes was also able to survive without any significant change during the storage of DCH with an a_w of 0.94 pressurized at 300 MPa (Trial 48; Table 2; Figure 4c). Compared to other trials, in this case, the survival of the pathogen could be favored by either (i) the high a_w value (0.94), since the application of a similar pressure level (347 MPa) in DCH with a lower a_w (0.919) resulted in the inactivation of the pathogen during the storage (Trial 41; Table 2; Figure 4b), and/or (ii) the lower pressure compared with trial 49 (at 450 MPa, a_w of 0.94) in which inactivation was observed. These hypotheses would be supported by Stollewerk et al. [24], showing that the levels of *L. monocytogenes* after the pressurization of smoked DCH (pH of 5.87 and a_w of 0.93) at 600 MPa for 5 min were low and progressively inactivated to limits below the quantification limit (0 log cfu/g) after the storage of the DCH at 4 °C for 38 days and afterwards at 8 °C for 18 days.

In DCH with an a_w of 0.919, *L. monocytogenes* decreased during the storage of DCH after HPP irrespective of the pressure level (from 347 to 600 MPa; trials 41, 42 and 43; Table 2; Figure 4b). Therefore, even if the pressure increase enhances the immediate lethality of *L. monocytogenes* during HPP, it has no effect on the inactivation of the pathogen during the subsequent storage after HPP in DCH with an a_w of 0.919.

Regarding the impact of fat, an increase from 18 to 42% did not significantly affect the *L. monocytogenes* lethality during HPP nor its subsequent inactivation after HPP in DCH with an a_w of 0.89 (trials 34–35; Table 2; Figure 4a). Contrarily, for DCH with an a_w of 0.95, a higher content of fat enhanced the lethality of *L. monocytogenes* by HPP and significantly reduced the inactivation of the pathogen during the subsequent storage (trials 52–53; Table 2; Figure 4d).

3.2.2. *L. monocytogenes* Growth during the Storage of DCH

In agreement with the observed results, *L. monocytogenes* growth was predicted to occur ($\Gamma > 0$) in products with $a_w \geq 0.96$ (trials 56–64; Table 2). *L. monocytogenes* was able to reach the maximum bacterial-population density (*ca*. 8 log cfu/g) even for DCH pressurized at 750 MPa (Figure 5).

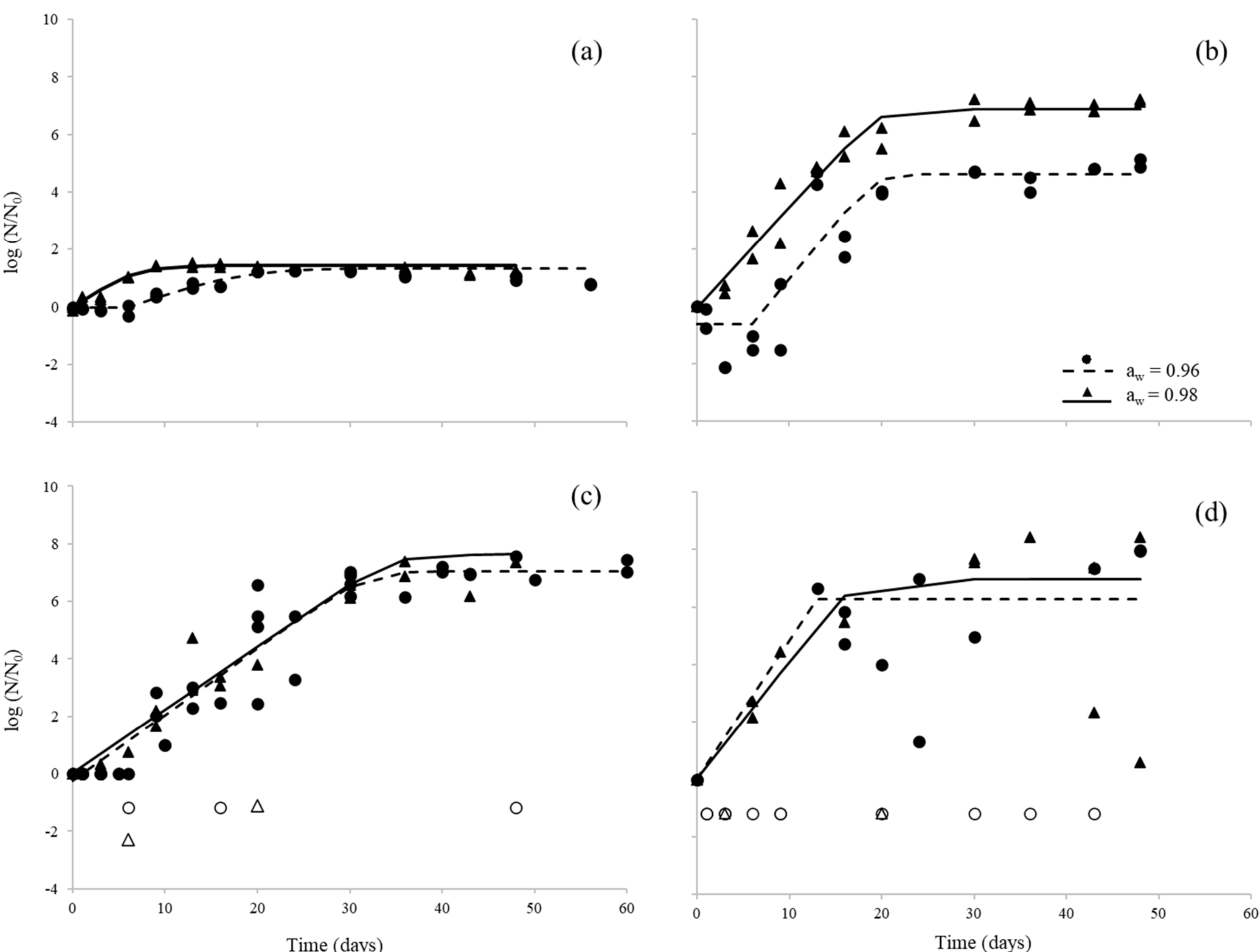

Figure 5. Growth of *L. monocytogenes* during storage at 7 °C of pressurized dry-cured ham (DCH) with an $a_w \geq 0.96$ pressurized at (**a**) 300 MPa (trials 56 and 61), (**b**) 450 MPa (trials 57 and 62), (**c**) 600 MPa (trials 58 and 63), and (**d**) 750 MPa (trials 59 and 64). Symbols correspond to log change values and lines to the fit of the Logistic growth model (Equation (3)) to data. Empty symbols correspond to replicates where the pathogen was not quantified but was detected after sample enrichment.

The growth kinetics were dependent on pressure level and DCH's a_w (which in turn determined the pathogen concentration at the beginning of the storage) (trials 56–64; Table 2). In this regard, the application of the lowest pressure level (300 MPa) caused little lethality. As a consequence, the high initial concentration (6.8–7.0 log cfu/g) of *L. monocytogenes* after HPP did not allow for proper estimation of the growth rate (trials 56, 61; Table 2; Figure 5a). The results show that DCH's a_w affected the lag time (λ) of *L. monocytogenes* in DCH pressurized at 300–450 MPa. The pathogen needed a λ of ca. 6 days before starting to grow in DCH with an a_w of 0.96 (trials 56–57; Table 2) but no λ was observed in DCH with an a_w of 0.98 (trials 61–62; Table 2). *L. monocytogenes* seemed to start growing immediately after the HPP at 600–750 MPa. The estimated growth rate was similar for DCH with an a_w of 0.96 and 0.98 (trials 58–60, 63–64; Figure 5c,d). Nevertheless, it has to be considered that at pressure levels of 600–750 MPa, variability in the *L. monocytogenes* concentration was observed between sample replicates along the storage time (i.e., from

7 log cfu/g to not detected), making the growth-kinetic-parameter estimates less accurate (Table 2; Figure 5c,d). Some works have associated this variability in the *L. monocytogenes* counts after HPP to the different injury degrees and/or different recovery capacities of the *L. monocytogenes* cells [27].

4. Conclusions

This study provides the identification and quantification of the growth/no-growth limits and the subsequent behavior of *Salmonella* and *L. monocytogenes* in pressurized DCH. This information can be relevant for producers of DCH, which can take advantage of it to adopt process and/or product criteria that can be implemented in the Hazard Analysis and Critical Control Point (HACCP) plan to manage the safety of their products. In this respect, the data provided in this study highlight the importance of considering the storage temperature as a criterion throughout the entire food chain, since it is the main factor not only inhibiting the growth but also favoring the non-thermal inactivation of mesophilic *Salmonella* in pressurized DCH. In the case of the psychrotrophic *L. monocytogenes*, producers of DCH can consider the identified growth/no-growth threshold value of the a_w in the final product as a product criterion to be implemented in the HACCP with the aim of proving that their products can be classified as RTE foods unable to support the growth of *L. monocytogenes*.

The impact of the combination of factors, e.g., the intrinsic and extrinsic characteristics of the products on the behavior of pathogens after HPP is diverse, highlighting the need to evaluate the microbiological risk associated with DCHs on a case-by-case basis. In this framework, the quantified inactivation or growth kinetics of *Salmonella* and *L. monocytogenes* in pressurized DCHs with different intrinsic characteristics can help producers of DCH to detect scenarios of particular risk comprising HPP lethality and/or the survival and growth of pathogens during storage. Moreover, producers of DCH can take advantage of the data provided in this study to enhance the safety of their products by designing strategies, such as the application of corrective storage before DCH commercialization, to reach the performance criteria for *Salmonella* and *L. monocytogenes* and thereby to enhance the compliance with regulatory, customer, and internal requirements during the shelf-life of DCH.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app122412732/s1, Figure S1: Detailed description of the modeling procedure for calculating the gamma (γ_X) values. Table S1: Cardinal parameters of *Salmonella* and *L. monocytogenes* used to estimate the growth/no-growth boundary through the gamma concept in the present study. Table S2: Gamma factors (γ), interaction term between factors (ξ), and overall gamma factors (Γ) calculated for *Salmonella* and *L. monocytogenes* according to the extrinsic and intrinsic characteristics of dry-cured hams (DCH) used in this study. Refs. [15,22,23,28–30] are cited in the Supplementary Materials.

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